Structural and Stereochemical Study of Galantinic Acid, a New Amino Acid from Peptide Antibiotic Galantin I

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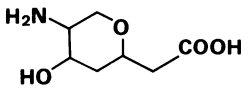
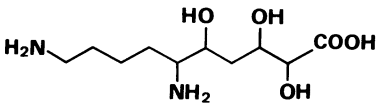
A new amino acid, galantinic acid, was found as one of the constituent amino acids in a peptide antibiotic galantin I. The structure of galantinic acid was determined to be (2S,4S,5S)-5-amino-2-carboxymethyl-4-hydroxytetrahydropyran on the basis of NMR and CD studies.

A peptide antibiotic galantin I effective against both Gram positive and Gram negative bacteria was isolated from the culture broth of *Bacillus pulvifaciensis* by Shoji and his co-workers.[1] Recently, we succeeded to determine the chemical structure of galantin I as shown in Fig. 1.[2]

From the hydrolyzate of galantin I, two new amino acids named galantinic acid (**1**) and galantinamic acid (**2**), were isolated and their primary structures had been preliminarily reported.[2] In the present paper, we describe the details about the structural determination as well as the assignment of steric configuration of galantinic acid.



1 galantinic acid



2 galantinamic acid

The isolation of galantinic acid from galantin I hydrolyzate was carried out by the use of ion-exchange chromatography and preparative paper electrophoresis. Galantinic acid was assumed to be a neutral amino acid taking into account the mobility on paper electropherogram and the retention time in amino acid analysis. However, its faster mobility or longer retention time compared with those of Gly or Ala indicated that galantinic acid would not be α-amino acid.

Characterization of galantinic acid was first achieved by NMR analyses. (Table 1) ^{13}C-NMR of this amino acid showed seven carbon signals which are of one carbonyl and six alkane carbons. Four of six alkane carbons were expected to carry heteroatoms such as *N* or *O* being classified into one carbon at higher field (51.8ppm) and three ones at lower field (64.7—73.6ppm). In general, the substitution of oxygen atom causes larger deshielding effect to the chemical shift of carbon atom than that of nitrogen atom. Therefore, we could deduce that amino group is attached on carbon atom at 51.8ppm and other three carbons are presumably bound to oxygen atoms.

^{1}H-NMR of galantinic acid revealed the presence of at least nine protons which are unexchangeable with deuterium. No protons were observed in olefinic or aromatic region. Of these protons, simple doublet centering at 2.39ppm (J=5Hz) was assumed to be methylene protons such as -X-C<u>H$_2$</u>-CH- (X=CO, NR$_2$, SR, *etc*). On the other hand, another seven protons appeared in complex multiplets which were expected from a ring structure. The correlations between carbon and proton signals were determined by the measurement of ^{13}C-NMR using selective decoupling method as summarized in Table 1.

Table 1. Correlations between ^{13}C and ^{1}H signals

δ		Supposed partial structure
^{13}C	Related ^{1}H	
37.6	1.43, 2.14	-C-<u>CH$_2$</u>-C-
41.6	2.39 (2H)	-C-<u>CH$_2$</u>-CO-
51.8	3.10	-C-<u>CH</u>-N-
64.7	3.44	-C-<u>CH$_2$</u>-O-, -C-<u>CH</u>-O- (×2)
67.2	3.82 (2H)	
73.6	4.11	
177.1	—	-C-<u>C</u>OOH

FD-MS spectrum of galantinic acid gave two peaks at m/z 175 and 176 which correspond to M^+ and $(M+H)^+$, respectively. When galantinic acid was *N*-benzoylated, the product gave two peaks at m/z 279 (M^+) and 280 ($(M+H)^+$) in FD-MS spectrum. This result clearly showed the introduction of one residue of benzoyl group indicating that galantinic acid is an aliphatic monoamino monocarboxylic acid.

From considerations of the molecular weight, carbon and proton numbers as well as the number of amino group, we could deduce unequivocally the molecular formula of galantinic acid to be $C_7H_{13}NO_4$. Therefore,

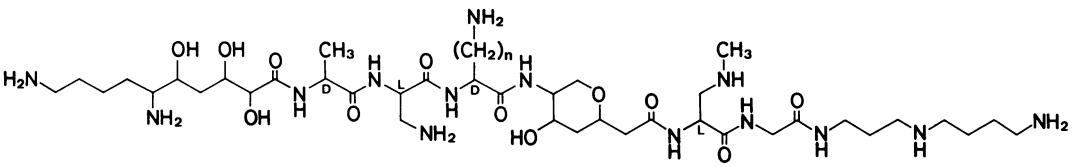


Fig. 1. The structure of galantin I. Galantin I is a mixture of two congeners differing at the fourth amino acid residue from N-terminal. Lysine (n=4) and ornithine (n=3) are found in a ratio of 9:1.

TABLE 2. ASSIGNMENTS OF PROTON SIGNALS AND THEIR SPIN COUPLING CONSTANTS (J/Hz)

δ	Multiplicity	Assignment	Coupled with							
			H_{2a}	H_{3a}	H_{3e}	H_{4a}	H_{5a}	H_{6a}	H_{6e}	H_7, H_7
			3.82	1.43	2.14	3.82	3.10	3.44	4.11	2.39
1.43	dt	H_{3a}	11	—	12	11	—	—	—	—
2.14	ddd	H_{3e}	2 or 5	12	—	5 or 2	—	—	—	—
2.39	d	H_7, H_7	7	—	—	—	—	—	—	—
3.10	dt	H_{5a}	—	—	—	11	—	11	4	—
3.44	t	H_{6a}	—	—	—	—	11	—	11	—
3.82	m	H_{2a} [a]	—	(11)	(2 or 5)	—	—	—	—	(7)
3.82	m	H_{4a} [a]	—	(11)	(5 or 2)	—	(11)	—	—	—
4.11	dd	H_{6e}	—	—	—	—	4	11	—	—

a) The analyses of multiplicity of H_{2a} and H_{4a} were impossible because of their complete overlapping. Therefore, the coupling constants only deducible from those of the related protons are shown in parentheses.

the degree of unsaturation of galantinic acid is two. Namely, one is of carboxylic acid and another one is of cyclic structure. The molecular formula also suggested that galantinic acid would be a hydroxyamino acid. When galantinic acid was oxidized with periodate and permanganate, no positive product to ninhydrin reaction was observed in amino acid analysis and on paper electropherogram. This result proposed the presence of vicinal amino alcohol moiety in galantinic acid.

Based on the facts mentioned above, a structure having tetrahydropyran skeletone was proposed for galantinic acid. The presence of three carbon atoms carrying oxygen atom, the multiplicity of proton signals, and their large coupling constants could be explained from the structure 1. The assignments of proton signals in ^{1}H-NMR (Fig. 2) were achieved by use of the decoupling method as summarized in Table 2. The position of each proton was exactly determined and the relative configuration of galantinic acid was then estimated by

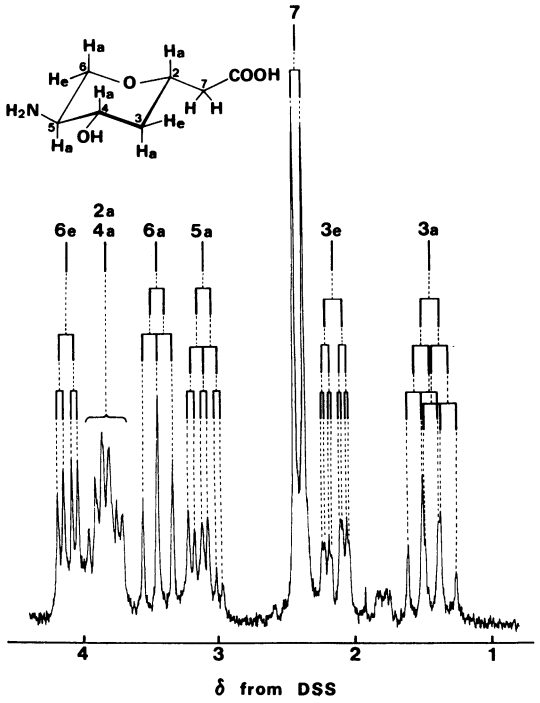


Fig. 2. ^{1}H-NMR of galantinic acid in D_2O (100 MHz).

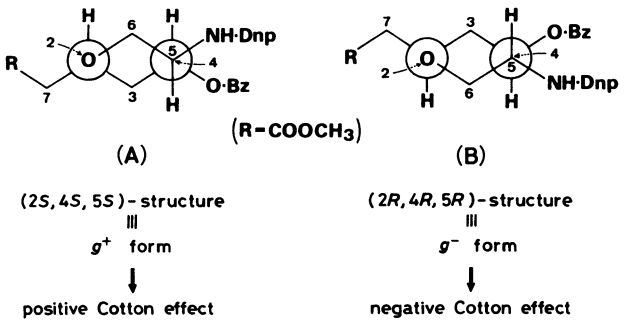


Fig. 3. Plausible stereo structures of galantinic acid and expected sign of Cotton effect of *N*-Dnp-*O*-Bz derivative. Dnp=2,4-dinitrophenyl; Bz=benzoyl.

precise analysis of the coupling constant of each proton. The fact that all the methine protons on C_2, C_4, and C_5 showed at least one large coupling constant over 10 Hz due to 1,2-diaxial protons might be elucidated from the steric structure mentioned in Fig. 2. For the purpose of determination of the absolute configuration of galantinic acid, Dnp-aromatic rule developed by Kawai and his co-workers was effectively applied.[3] The absolute configuration of vicinal amino alcohol part is thus deducible from the sign of CD spectrum of *N*-Dnp and *O*-Bz derivative. According to this rule, the structure **A** (g^+ form) of the two plausible stereo structures (Fig. 3), would show a positive Cotton effect, while the

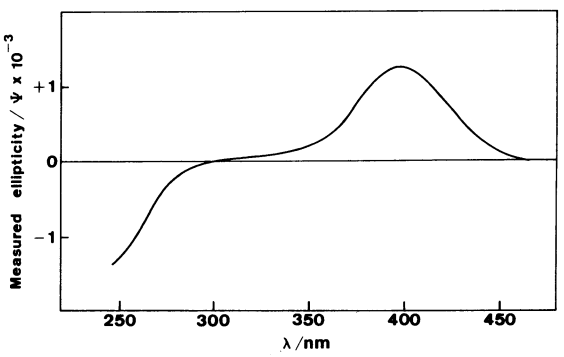


Fig. 4. CD spectrum of *N*-Dnp-*O*-Bz-galantinic acid methyl ester obtained in $CHCl_3$. The Cotton effect around 240 nm was not observed because of the disturbance due to solvent absorption.

structure **B** (g^- form) which corresponds to an enantiomeric form of the structure **A** would be expected to show a negative Cotton effect around 400nm in CD spectrum.[4] *N*-Dnp-*O*-Bz-galantinic acid methyl ester prepared from the natural sample gave clearly a positive Cotton effect suggesting the g^+ form. (Fig. 4) As a result, the absolute structure of galantinic acid was defined to be (2*S*,4*S*,5*S*)-5-amino-2-carboxymethyl-4-hydroxytetrahydropyran.[5]

Experimental

The NMR spectra were obtained with Varian XL-100-15 and JEOL JNM-FX-90Q spectrometers for ^1H and ^{13}C in D_2O, respectively. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as the external standard and the chemical shift were given in δ value (ppm) from the standard. FD and EI mass spectra were measured with a JEOL JMS-O1SG-2 mass spectrometer. CD spectra were recorded with a JASCO ORD/UV-5 spectrometer in $CHCl_3$.

Isolation of Galantinic Acid. Crude galantin I (0.44g) was hydrolyzed for 5h at 110°C with 10ml of constant boiling 6M† hydrochloric acid in a sealed tube after deaeration. The hydrolyzate obtained from 1.76g of galantin I was concentrated *in vacuo* and the residue was applied to Amberlite IRCG-50 column (type I, NH_4^+ form, 1.5×140cm). The elution was carried out with a gradient method from 0.1% to 1.5% aqueous ammonia. Thus, a mixture of neutral amino acids was obtained in a yield of 0.21g.

The mixture of neutral amino acids (1.4g) obtained by the above procedure was dissolved in a small amount of 1M HCl and 95% ethanol was added to the solution. The precipitate was deposited by centrifugation and the supernatant was concentrated *in vacuo*. A powdery residue obtained was applied to Amberlite IRCG-120 column (type I, pyridine form, 1.8×50cm) which was eluted with a linear gradient method from 0.125M to 0.50M pyridine-acetate buffer (pH 3.5). The fractions containing galantinic acid were pooled and concentrated *in vacuo*. The residue was dissolved in a small amount of 1M HCl and ethanol was added. The supernatant after centrifugation was concentrated *in vacuo* and the residue was purified by preparative paper electrophoresis (pyridine-acetic acid-water=1:10:89, pH 3.5, 13V/cm, 2h, Toyo Roshi No. 50 filter paper). Galantinic acid was extracted from the paper with 0.1M acetic acid. The extract was lyophilized and the lyophilization was repeated twice after dissolving the residue in a small amount of water. Pure galantinic acid (26mg) was obtained as hygroscopic powder which showed a single spot on paper electropherogram and TLC (cellulose plate, developing solvent: *n*-BuOH-pyridine-AcOH-H_2O=15:5:8:12, R_f 0.68).

N-Benzoylgalantinic Acid for FD-MS Measurement. To a solution of galantinic acid (1.0mg, 5.7μmol) in 0.1ml of water were added benzoyl chrolide (2.1mg, 15μmol) and 4.8% Na_2CO_3 solution (0.05ml, 22.5μmol). The reaction mixture was stirred overnight at room temperature and then once extracted with ether to remove exess benzoyl chloride. The aqueous layer was acidified with hydrochloric acid and extracted with ethyl acetate. The extract was washed with water, dried over $MgSO_4$ and then concentrated *in vacuo*. A solution of the residue in 0.1ml of DMSO was subjected to FD-MS measurement: *m/z* 279 (M^+) and 280 ($(M+H)^+$).

N-(2,4-Dinitrophenyl)-O-benzoylgalantinic Acid Methyl Ester. To a solution of galantinic acid (1.0mg, 5.7μmol) in 50μl of H_2O were added $NaHCO_3$ (1.4mg, 17μmol) and 0.5mM 2,4-dinitrofluorobenzene (DNFB) in acetone (34μl, 17μmol). The mixture was stirred for 4h at room temperature in the dark. The reaction was followed by TLC (silica gel plate, developing solvent: $CHCl_3$-MeOH-AcOH=6:2:1). Extra reagents (0.4mg of $NaHCO_3$; 10μl of DNFB in acetone) were added every a few hours until the starting material disappeared.

After the completion of the reaction, the mixture was extracted with ether several times and the aqueous layer acidified with 2M HCl was concentrated *in vacuo*. The residue in ethyl acetate was washed with saturated NaCl solution several times and organic layer was dried over $MgSO_4$. To the solution was added an excess of CH_2N_2 in ether. The mixture was allowed to stand for 1h and then concentrated *in vacuo*.

To the residue in CH_2Cl_2 (0.2ml) were added benzoic anhydride (10mg, 44μmol), triethylamine (6.1μl, 44μmol), and 4-dimethylaminopyridine (0.3mg, 2.5μmol). The mixture was stirred overnight at room temperature in the dark and then subjected to preparative TLC. The reaction mixture was applied to silica gel plate (Merck, 0.25mm thickness, 20×20cm) which was developed with benzene-ethyl acetate (4:1). Yellow band corresponding to the desired product was extracted with $CHCl_3$ and the extract was concentrated *in vacuo*. The crystalline residue thus obtained gave a peak at *m/z* 459 (M^+) in both FD and EI mass spectra. This sample was used for the measurement of CD spectrum.

Periodate-permanganate Oxidation of Galantinic Acid. Galantinic acid (0.43mg) was dissolved in 0.05M sodium acetate buffer (pH 7.7, 200μl). To the solution was added 0.1M sodium periodate solution (100μl). The mixture was allowed to stand for 2d at 4°C in the dark and then 0.2% potassium permanganate solution was slowly added until a pale purple color remained. MnO_2 formed was removed off by centrifugation. The supernatant was subjected to an amino acid analysis and a paper electrophoresis.

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3) a) M. Kawai, U. Nagai, M. Katsumi, and A. Tanaka, *Tetrahedron*, **34**, 3435 (1978); b) M. Kawai, U. Nagai, and A. Tanaka, *Bull. Chem. Soc. Jpn.*, **55**, 1213 (1982).

4) (1*R*,2*R*)-2-Aminocyclohexanol, an example of g^- form, showed a negative Cotton effect. (Private communication from Dr. M. Kawai.) Abbreviations: Dnp=2,4-dinitrophenyl; Bz=benzoyl; g^+ and g^-=*gauche* forms possessing positive and negative bond chirality, respectively.

5) Synthetic (2*S*,4*S*,5*S*)-compound was completely identical with natural product in all respects. (Private communication from Dr. Y. Ohfune)

† 1 M=1 mol dm^{-3}.